INFECTION AND IMMUNITY, June 1993, p. 2296–2303
0019-9567/93/062296-08$02.00/0

Vol. 61, No. 6

Development of a Trehalose 6,6′-Dimycolate Model Which Explains Cord Formation by *Mycobacterium tuberculosis*

CYNTHIA A. BEHLING,[1]† BETH BENNETT,[2] KUNI TAKAYAMA,[3,4] AND ROBERT L. HUNTER[2]*

Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia 30322[1,2]; Mycobacteriology Laboratory, William S. Middleton Memorial Veteran's Hospital, Madison, Wisconsin 53705[3]; and Department of Bacteriology, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706[4]

Received 12 November 1992/Accepted 29 March 1993

Trehalose 6,6′-dimycolate (TDM) is a glycolipid of mycobacteria that displays an unusual toxicity and has been reported to be a virulence factor for *Mycobacterium tuberculosis*. Lack of understanding of the toxicity has impeded acceptance of TDM as a virulence factor. We previously reported that the toxicity of TDM depends on its presentation as a surface monolayer consisting of 30% trehalose and 70% exposed mycolic acid moieties. This paper further investigates the structure of the TDM monolayer. It began with the observation that beads coated with TDM, but not with closely related analogs, aggregate to form organized structures resembling the cords of virulent mycobacteria. This implied that the TDM molecules in the monolayer were arranged in an organized structure. This structure was investigated by real-time kinetic microscopy, scanning electron microscopy, and gross observations of the adhesion patterns of TDM-coated beads. In each of these models, the structures induced by TDM differed from those of analogs or other amphiphiles studied. These observations were used to construct a model of the structure of TDM monolayer which envisions linear arrays of TDM molecules arranged in a circumferential pattern on beads with discontinuities only at the two poles.

Trehalose 6,6′-dimycolate (TDM) has a long and controversial history as a virulence factor for *Mycobacterium tuberculosis*. Robert Koch first noted that *M. tuberculosis* organisms grown in culture aggregate to form rope-like structures now known as cords (10). A half-century later, Middlebrook, Dubos, and Pierce proposed that cord formation was due to a substance on the surface of mycobacteria and that it was an "essential accompaniment of virulence" (14). In 1950, Hubert Bloch identified a toxic glycolipid on the surface of virulent *M. tuberculosis* organisms which he termed "cord factor" (1). Purified cord factor was later identified as TDM (15). TDM has been studied extensively as an immunomodulatory agent for inducing tumor regression and as a vaccine adjuvant (8, 11–13, 20, 23, 24, 26). Its most unusual property is an acute, nonallergic toxicity that is not dose related (1, 2, 27). One injection of 10 μg with mineral oil induces hemorrhagic pneumonitis, and three injections are lethal to C57 black mice. The oil, by itself, is innocuous. In contrast, 1,000-fold larger doses of TDM injected without oil elicit no overt toxicity (1).

Several lines of evidence strongly suggest that TDM is necessary for the proliferation and/or virulence of *M. tuberculosis* and *Mycobacterium bovis* BCG (BCG) in vivo. The most direct evidence, published by Silva et al., demonstrated that removal of TDM from the surfaces of BCG cells reduces their ability to persist in the lungs and that restoration of TDM to the surfaces of the organisms restores their ability to persist in vivo (21). In addition, Bloch and Noll reported that injections of doses of TDM in mineral oil too small to cause toxic manifestations by themselves enhanced both acute and chronic tuberculosis in mice and guinea pigs and caused the animals to die within shorter periods of time with massive infections (2). The effect appeared to be specific. TDM failed to influence the course of infections other than tuberculosis, and closely related mycobacterial lipids had no effect on tuberculosis (2). In earlier studies, Bloch had reported finding high concentrations of cord factor on virulent (strain H37Rv) organisms but little on the avirulent ones (strain H37Ra) (1). He demonstrated that removal of cord factor from *M. tuberculosis* organisms reduced their ability to cause progressive infections in mice. Finally, susceptibility to the toxicity of TDM among strains of mice correlated with susceptibility to mycobacterial infection. The C57 black and Dba strains were highly susceptible to tuberculosis and to the toxicity of TDM in oil, while Swiss albino and CF1 strains were resistant to both (1).

These data seemed to provide compelling evidence that TDM contributes to the virulence of human and bovine tuberculosis. However, other data complicated the picture. First, there is no correlation between the amount of TDM produced by an organism and its virulence (8, 27). TDM is found in nearly all mycobacteria, and large amounts are found in certain avirulent organisms such as *Mycobacterium smegmatis*. Second, the requirement that TDM be injected with oil to enhance mycobacterial infections or to induce severe toxic reactions was not explained. The opinion of most observers for over 3 decades was expressed by Youmans: "Cord factor, therefore, exhibits some almost unique biologic activities that may ultimately secure for it the distinction *of being a virulence factor for tuberculosis*. However, because of the unnatural requirements (oily solutions or their aqueous emulsions) for expression of its biologic activities, . . . our endorsement of this view is, at best, reserved" (27) [material in italics is paraphrased].

In 1981 and 1982, Retzinger and associates published an explanation of these unusual properties of TDM (18, 19). They recognized that as an insoluble amphiphile, TDM would preferentially localize at hydrophobe-water interfaces. They demonstrated that TDM formed a rigid insoluble

* Corresponding author.
† Present address: Department of Pathology, University of California at San Diego, San Diego, CA 92124.

monolayer at such interfaces and proposed that its toxicity was due to its presentation and conformation on hydrophobic surfaces (19). This hypothesis was revolutionary in that toxic surface mechanisms had been previously recognized for crystals such as silica and monosodium urate but not for microbial products or lipids (25). The hypothesis predicted that the orientation and surface area occupied by TDM on interfaces rather than the dose would correlate with toxicity.

A quantitative method for studying surface layers of TDM was developed by replacing oil drops with hydrophobic beads (18, 19). As a molecular monolayer on beads, TDM was found to induce all of the toxic reactions characteristic of oil emulsions, including granulomas, hemorrhagic pneumonitis, and death of C57 black mice (18). The intensity of the responses was, indeed, proportional to the surface area of the molecular monolayer of TDM. Identical or larger doses of TDM injected in forms other than the monolayer were biologically inert. The responses persisted as long as the monolayer of TDM remained on the beads in vivo.

Direct measurements of the TDM monolayer at the air-water interface demonstrated that hydrophobic mycolic acids occupied 70% of the total surface area while the trehalose head groups occupied the remaining 30% (19). Further studies produced similar measurements of monolayers on hydrophobic beads. The equilibrium spreading area of TDM on beads in aqueous media was determined by isotope counts and protein adhesion methods to be between 0.15 and 0.2 $\mu g/cm^2$ (19).

The present studies were initiated by the observation that TDM-coated beads aggregated in a peculiar way to form elongated structures reminiscent of cords of *M. tuberculosis*. Since this phenomenon was not predicted by previous studies, we undertook further investigation of the structure of the TDM monolayer and its biologic activities. This manuscript reports physical-chemical studies with TDM and several related analogs. It provides evidence for a revised model of the TDM monolayer and an explanation for the formation of cords. Our hypothesis is that study of the structure and biologic activities of surface layers of TDM will lead to advances in the understanding of TDM toxicity and the pathogenesis of tuberculosis.

MATERIALS AND METHODS

Mycobacterial lipids. TDM and the other glycolipids (trehalose monomycolate, galactose-galactose dimycolate, trehalose dipalmitate, trehalose dicorynomycolate, and mannose-mannose dimycolate) used in these studies were prepared by Mayer Goren and colleagues as previously described (7). The chemical structures and biologic activities of TDM from various bacteria have been well characterized (11). The mycolic acid moieties of TDM differ from strain to strain and to a smaller extent within strains of bacteria. Mycobacterial mycolic acids range usually from 60 to 90 carbon atoms. Other bacteria, such as *Nocardia* species, produce smaller mycolic acids. TDMs with mycolic acid chain lengths comparable to those of *M. tuberculosis* have biologic activities which are similar to those from virulent mycobacteria (8, 11). The TDM from *M. smegmatis* used in these studies was similar to that from *M. tuberculosis* (7, 22).

Coating of beads. Styrene divinylbenzene beads (Duke Scientific Corp., Palo Alto, Calif.) with mean diameters of either 1.0 or 5.7-μm were cleaned and coated with TDM as previously described (18, 19). Briefly, the beads were coated with TDM by mixing them with TDM dissolved in hexane-ethanol (9:1) and then rapidly evaporating the solvent under a stream of nitrogen with vigorous agitation. The TDM is deposited as an even layer on the beads. The beads were suspended in phosphate-buffered saline. The beads were coated with either 0.3 or 4.0 μg of TDM per cm^2. The amount was calculated to have surface layers of TDM between 1 and 2 molecules thick or greater than 10 molecules thick. Beads (mean diameter, 1 or 5.7 μm) were coated with trehalose monomycolate and galactose-galactose dimycolate in the same manner.

Microscopic studies. A method for viewing defined layers of glycolipids at a hydrophobe-hydrophile (air-water) interface was developed. A 0.25-cm^2 silica chip was cleaned by sonication in Freon 113 for 15 min. A 10-μl drop of deionized water was placed on its surface. A measured amount of TDM or other amphiphile was dissolved in 10 μl of hexane-ethanol (9:1). This solvent was then carefully placed onto the water drop with a micropipette. The solvent evaporated, and the resulting surface structures were viewed under light microscopy with a reflected, high-intensity light source. Since air is hydrophobic, the surfactant monolayers in this preparation were inverted compared with those on beads (18).

Scanning electron microscopy (SEM) studies of the aqueous surfaces of TDM on beads were carried out by a procedure previously developed in our laboratory for viewing the surfaces of oil-in-water emulsions (5). Protein casts of the bead surfaces were viewed in the upper stage of an ISI DS-130 SEM. The casts were formed with TDM-coated beads embedded in a 50% bovine serum albumin solution and fixed with 25% glutaraldehyde. The pellet was critical-point dried, fragmented, and sputter coated with gold as previously described (5). This procedure allowed observation of the surfaces of the beads or of casts of the bead surfaces.

RESULTS

Gross and microscopic appearances of beads coated with TDM. Plain styrene divinylbenzene beads or beads coated with a molecular monolayer (0.3 $\mu g/cm^2$) of TDM were suspended in normal saline in a conical tube. Uncoated beads settled into a pellet and remained at the bottom of the tube (Fig. 1A). Beads coated with a monolayer of TDM slowly climbed the walls of the tube (Fig. 1B). They formed a lacy veil which moved freely from side to side as the tube was gently rotated. This indicated that the beads were not adherent to the sides of the tube. If the tube was shaken, the veil disintegrated and the beads settled to the bottom. Over the next several days, they again climbed the walls of the tube to form a similar veil. This process could be repeated many times. Beads coated with a surface excess of TDM (4.0 $\mu g/cm^2$) also climbed the walls of the tube but formed a coarse pattern of larger aggregates which adhered tightly to the walls of the tube (Fig. 1C). They could be removed only with vigorous agitation or sonication.

Microscopically, the veil formed by beads coated with a monolayer of TDM was composed of linear aggregates which resembled serpentine cords of mycobacteria (Fig. 2B). Beads coated with a 10-fold surface excess of TDM aggregated avidly and formed cords, but the aggregates were less regular, had more branches, and were larger than those formed by beads coated with a monolayer of TDM. Uncoated beads formed small random aggregates in distilled water and a nearly unidispersed suspension in saline (Fig. 2A). The cords of TDM monolayer-coated beads were seldom single chains of particles, such as those formed by

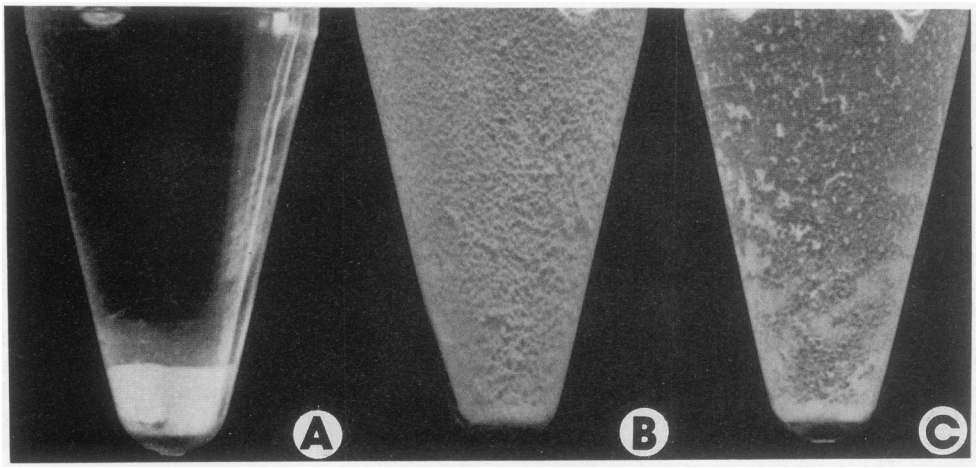

FIG. 1. Climbing veil of beads coated with a monolayer or multilayers of TDM. A total of 10^8 beads in 15-ml conical-bottom polystyrene tubes were allowed to stand at room temperature in saline for several days. (A) Uncoated beads; (B) beads coated with a monolayer (0.3 μg of TDM/cm^2); (C) beads coated with an excess (4.0 μg of TDM/cm^2).

streptococci, but typically had diameters between two and three beads thick. The cords packed together in a tangle. Gentle pressure on the coverslip disrupted the tangles into individual cords which contained only occasional branches.

Studies to evaluate the time course and mechanism of cord formation were done. Cords of TDM monolayer-coated beads were sonicated to produce a unidispersed suspension. These beads reformed cords within minutes, with the result that the process could be observed with a microscope. Cord formation appeared to be the result of oriented bead-to-bead interactions rather than random collisions. Beads or fragments of cords would drift close together and then rotate to some preferred position and appear to suddenly snap together. Cords formed by TDM monolayer-coated beads were exposed to 1% Tween 80 or 5% ethanol to evaluate the nature of the adhesive force between beads. A drop of ethanol or Tween 80 solution was placed adjacent to a drop of corded beads suspended in saline. The cords were rapidly disrupted by either of these agents as soon as the drops made contact. This suggested that the adhesive force between the beads was a hydrophobic interaction.

Similar studies were done with beads coated with trehalose monomycolate, galactose-galactose dimycolate, trehalose dipalmitate, trehalose dicorynomycolate, and mannose-mannose dimycolate. None of these analogs of TDM was able to induce the formation of cords like those induced by a monolayer of TDM. In general, these aggregates tended to be random clusters of beads rather than the oriented cords formed by TDM-coated beads. Trehalose dicorynomycolate-coated beads formed small, tight aggregates. Mannose-mannose dimycolate-coated beads formed large, irregular branching aggregates. It may be noted that the toxicities of the TDM diastereomers were notably attenuated (7).

Light microscopic studies at the air-water interface. Observations of glycolipids at the air-water interface provided direct evidence that TDM forms a unique surface structure. TDM dissolved in hexane-ethanol was placed on the surface of a water drop and viewed under a dissecting microscope.

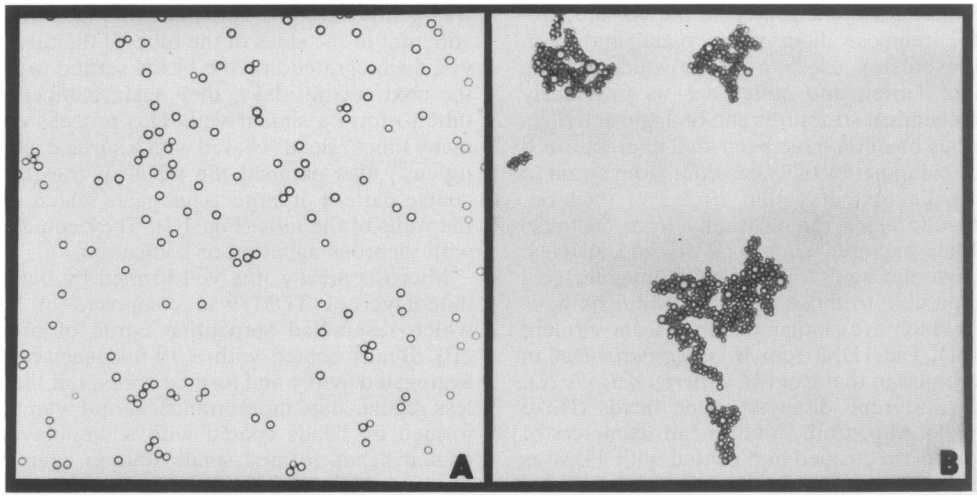

FIG. 2. Cord formation by coated beads. Beads coated with a monolayer of TDM form characteristic linear aggregates when suspended in saline. (A) Uncoated beads; (B) beads coated with a monolayer of TDM.

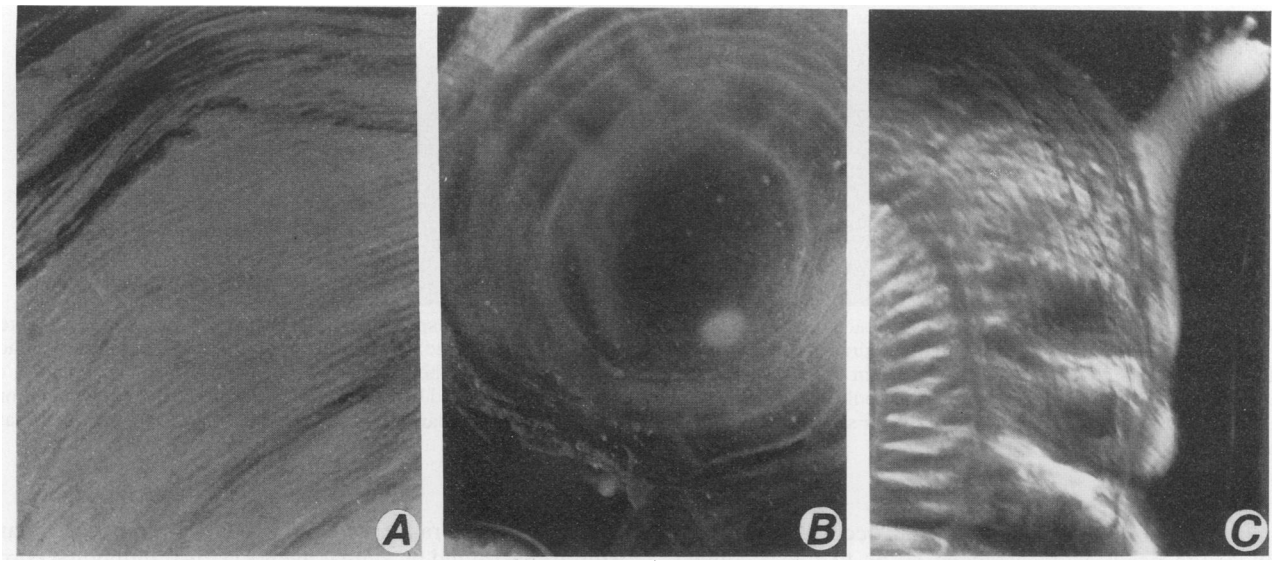

FIG. 3. TDM at the air-water interface. TDM was dissolved in hexane-ethanol (9:1) and evaporated onto a 10-μl water drop placed on a silica chip. The solvent evaporated to form the characteristic structures shown here at a magnification of ×64. (A) TDM at 10 times the monolayer concentration at 1 min; (B) TDM at 50 times the monolayer concentration at 1 min, showing increased thickness of the circumferential lines; (C) TDM at 10 times the monolayer concentration at 15 min, showing the effects of compression due to evaporation of water. The TDM layer shows buckling perpendicular to the lines of TDM.

The TDM formed a highly organized pattern of concentric circles over a period of 2 min as the solvent evaporated. When a monolayer amount of TDM dissolved in hexane-ethanol was placed on the surface, the lines were barely visible. When more TDM was added, the existing lines were dissolved by the solvent and replaced by thicker, more visible ones as the solvent evaporated. The finest lines visible had a periodicity of approximately 10 μm (Fig. 3A). As more TDM was added, additional thicker, less-regular lines appeared (Fig. 3B). These thicker lines were seen to be composed of compressed thinner lines. They were always oriented in a circumferential fashion around the dome of the water drop. As the solvent evaporated, the pattern buckled in a direction perpendicular to the linear structures (Fig. 3C). This is evidence of an insoluble layer of surface lipid that is compressible in the direction parallel to its primary orientation but that is incompressible in the direction perpendicular to it.

Similar studies were performed under identical conditions with analogs of TDM in which the sugar moiety, length, number, or type of hydrocarbon chain was varied. In addition, dodecanoic acid, stearic acid, several synthetic block copolymer surfactants, and lipid A were examined. Each amphiphile produced a characteristic pattern on the surface of water. None produced a pattern resembling the concentric rings of TDM. As expected, soluble amphiphiles, such as lipid A, did not induce any visible irregularities on the surfaces of water drops. Fatty acids and insoluble block copolymers aggregated to form discrete clumps but formed no organized pattern.

Trehalose monomycolate initially formed fine linear structures similar to those of TDM, but they were less well defined and less stable. Over a few minutes, the lines disintegrated by thinning in some areas and thickening in others to form a beaded pattern. If more trehalose monomycolate was added, larger clumped aggregates rather than the linear structures characteristic of TDM formed. Mannose-mannose dimycolate formed fine structures that were unstable. Within seconds they disintegrated into beaded aggregates. The galactose-galactose dimycolate analog of TDM was surprisingly different. Galactose-galactose dimycolate is an isomer of TDM which differs only in the orientation of a single hydroxyl group. Nevertheless, this small change is sufficient to induce the formation of fragile linear structures which rapidly coalesced at the edge of the water drops and disintegrated. Trehalose dicorynomycolate, which is identical to TDM except for having shorter fatty acid chains, formed a completely different pattern. It initially formed small needle-like aggregates which rapidly coalesced at the edge of the water drops. There was no tendency to form linear structures, even transiently. Trehalose dipalmitate formed structures similar to those of trehalose dicorynomycolate. The TDM monolayer, consequently, required a specific geometry of both the hydrophobic fatty acid and hydrophilic trehalose moieties. If either the fatty acid or sugar moiety was changed, the characteristic TDM surface structure failed to form.

SEM studies. SEM was used to further evaluate structures formed by TDM on the surfaces of hydrophobic beads. Because surface structures of amphiphiles are characteristically dependent on the hydrogen-bonding activity of water, a protein-casting SEM procedure was developed to study the aqueous surfaces of insoluble amphiphiles (5). Freeze-fractured preparations of this type revealed beads protruding from the protein matrix or casts of beads in the matrix. Plain uncoated beads had smooth surfaces in all preparations within the limits of resolution of the microscope. Beads coated with a nominal monolayer concentration (0.3 $\mu g/cm^2$) of TDM exhibited rings which formed a roughly circular pattern with an equator and a pole (Fig. 4A and B). Frequently, a button of extra material was present at the center of the circles at the pole of the bead (Fig. 4A). Casts of the bead surface provided finer resolution. They demonstrated a denser accumulation of lines which were always arranged in a circumferential fashion around the beads (Fig. 4C). Fragments of cords were observed with sufficient resolution to

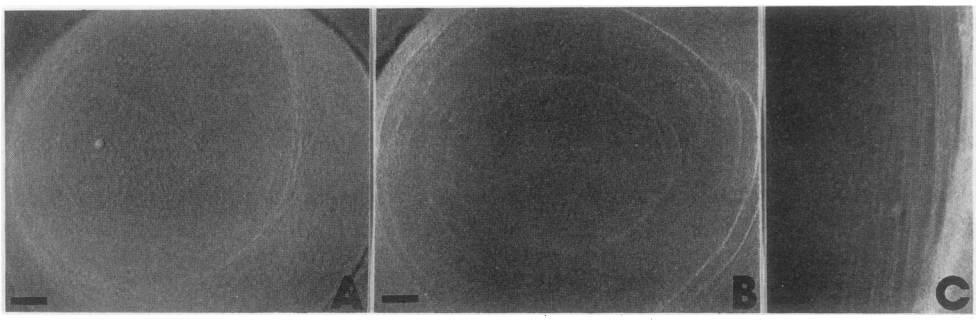

FIG. 4. Ultrastructure of TDM on beads. Beads coated with a monolayer of TDM were suspended in 25% bovine serum albumin and fixed with glutaraldehyde. The block was then dehydrated, frozen in liquid nitrogen, and fractured. The fracture face, containing exposed beads and bead casts, was coated with gold-palladium and examined by SEM. (A) TDM coated-bead with circumferential lines and a button of material thought to be aggregated TDM at the pole (magnification, ×15,200); (B) TDM-coated bead with circumferential lines (magnification, ×15,200); (C) cast of a bead showing finer linear structures (magnification, ×30,500). Scale markers in panels A and B and in panel C indicate 500 and 250 nm, respectively.

evaluate surface structures on several occasions. In each instance, the beads were arranged so as to make contact along the circumferential lines formed by lines of TDM.

DISCUSSION

With the resurgence of mycobacterial infections, especially drug-resistant tuberculosis, there is a renewed need for an increased understanding of factors which contribute to virulence. There has long been evidence that TDM can potentiate mycobacterial infections. There is even evidence that it influences susceptibility to antibiotics. Small doses of TDM potentiated infection of mice with tuberculosis and caused them to die of rapidly progressive infections even in the face of otherwise adequate therapy with isoniazid (3). In spite of these dramatic results, the requirement for oil and the lack of dose-response relationships placed research on TDM outside mainstream science and engendered controversy. Retzinger's demonstration that the toxicity of TDM depends on its presentation as a surface monolayer provided an explanation for these inconsistencies and a model for further investigation.

The present study was initiated to investigate the structure of the TDM monolayer and to determine its dependence on the structure of the glycolipid. It began with the demonstration that TDM can induce particles to aggregate in a pattern reminiscent of the cords of *M. tuberculosis* organisms. Cord factor was named because its removal from the surface of mycobacteria disrupted cords (1). Its ability to induce cords had never been demonstrated prior to our studies (27). TDM was dissolved in a mixture of hexane and ethanol before being placed on the beads or surfaces of water drops. Since hexane may dissolve in bilayer membranes, this solvent may have influenced the structures formed by TDM. We believe that this solvent effect was not critical because the monolayer remained stable under conditions that would be expected to remove most solvent, namely, lyophilization of beads and storage for 6 months at room temperature (15). In addition, beads coated with TDM from solution in ether had physical and biologic properties identical to those that were coated with TDM dissolved in hexane-ethanol.

Several studies were done to evaluate the mechanism of cord formation and to determine whether it could be similar to that of cord formation of mycobacteria. Disruption of the cords by alcohol and nonionic surfactants confirmed that the aggregation of beads induced by TDM was mediated by hydrophobic interactions. Cords of *M. tuberculosis* organisms are disrupted by similar agents (1, 14). Individual cords of beads, like those of mycobacteria, occasionally reached a length of several hundred microns. By these criteria, the cords formed by TDM-coated beads were similar to those formed by mycobacteria. The structure of the cords also provided information about the underlying orientation of TDM molecules. The formation of long linear aggregates implies a high degree of directional orientation of the adhesive domains on individual beads. Randomly oriented adhesive domains typically produce random aggregates. The most regular cords formed when the beads were covered with a monolayer of TDM. This argued against Retzinger's hypothesis that cords were induced by surface excess TDM which formed micellar structures (19). It also implied that contact of TDM with the hydrophobic surfaces of the beads was required for the formation of the oriented adhesive monolayer.

A most intriguing property of beads coated with a monolayer of TDM was their ability to form a meshwork of cords which climbed the inclined walls of plastic tubes as a lacy veil. They would reclimb the walls of the tubes many times after being shaken down. Hydrophobic adhesion was the only source of energy available to the beads for such climbing. The laws of physical chemistry dictate that once contact is established between the beads, forces act to minimize the surface free energy by maximizing the area of contact between hydrophobic domains on adjacent beads. We propose that the energy of rotation and realignment of the adhesive domains caused the veil of beads to expand and climb. This rotation and realignment was observed by light microscopy. The beads did not immediately stick where they first touched but moved or rotated to an aligned position before adhering.

Real-time kinetic light microscopy of the formation of structures at the air-water interface provided further evidence of the uniqueness of the surface structures. TDM, but not any of the analogs, produced rigid arrays of stable circular lines. This is consistent with the proposed model of a highly organized two-dimensional crystalline structure. Small changes in molecules such as optical isomers typically induce major changes in crystal structure. The molecular stereochemical properties of trehalose responsible for its ability to form stable structures at hydrophobic interfaces are well known (17). The ability of trehalose to substitute for water and protect organisms from drying is thought to be

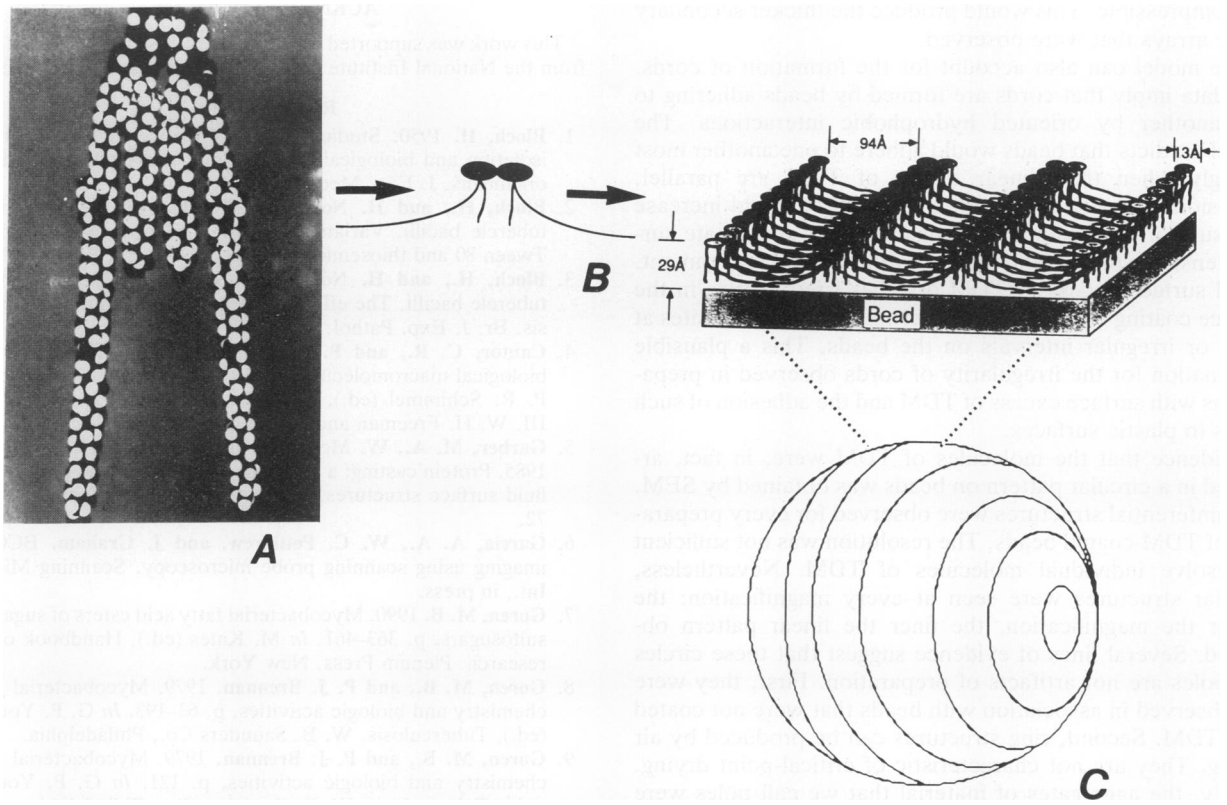

FIG. 5. Model of the TDM monolayer. In this model, TDM forms linear arrays of trehalose head groups separated by troughs of exposed mycolic acids. Compression by forces perpendicular to the linear array would result in the linear structures as seen on the bead surfaces. Alteration of the sizes of the sugar head groups, the positions of the mycolic acid chains, or the lengths or types of hydrocarbon chains would be expected to significantly change the monolayer. (A) Space-filling model of a TDM molecule; (B) schematic drawing representing a TDM molecule; (C) schematic model of a TDM monolayer on the surface of a hydrophobic styrene divinylbenzene bead.

critical for the survival of certain microbes and insects (16). It seems likely that these properties of trehalose contribute to the structure of the TDM monolayer. As the water evaporated and the TDM surface was compressed, it buckled only in a plane perpendicular to the primary lines, demonstrating that the surface was rigid in one direction and compressible in another.

The question of how molecules of TDM might be arranged on the surfaces to produce the characteristic structure and the adhesive properties of coated beads was approached by modeling. Several pieces of information were available. The dimensions of the TDM molecule, including the size of the trehalose head group and the length of the hydrophobic chains, were known from the chemical structure. The work of Retzinger provided measurements of the equilibrium spreading area occupied per molecule of TDM on a monolayer (172 $Å^2$) and the percents of a monolayer composed of exposed trehalose (30%) and of hydrophobic mycolic acid domains (70%) (19). Studies of the adhesion of proteins, especially fibrinogen, suggested that hydrophobic domains measured 90 Å in either width or diameter. Electron micrographs of cylindrical micelles produced by Retzinger demonstrated linear structures with a periodicity of approximately 90 Å (19). The formation of cylindrical micelles is a rare property of amphiphiles (4). It implies a particular wedge-like geometric molecular shape.

The key departure of the present study was the observation that beads coated with a monolayer, but not multilayers, of TDM formed cords and that the process of cord formation released energy sufficient to cause the beads to climb the walls of a conical plastic tube. These phenomena were not predicted by the Retzinger model. The formation of linear aggregates by TDM-coated beads implied an underlying linear orientation of the arrays of TDM molecules. A simple alignment of TDM molecules with the long hydrocarbon chains spread to either side was consistent with all of the measurements described above. After construction of a number of different models, it became evident that the simplest and most satisfactory arrangement was one in which linear arrays of TDM molecules were simply wound around the beads like string on a ball (Fig. 5). The parallel linear arrangement of hydrophilic and hydrophobic domains could be maintained without compression or distortion except for two points at the poles. The light and electron microscopy observations were made after formulation of this model, and they serve to add credence to it.

The model accounts for the observed structure of TDM layers at the air-water interface. This is essentially a two-dimensional crystalline structure. Changes in either the sugar or fatty acid moieties would be expected to have major influences on the packing of molecules within such a crystalline structure. Pressure on the crystalline layer of TDM parallel to the linear arrays would be transmitted through closely packed sugar groups which are not compressible. The layer would warp and eventually buckle rather than compress. Pressure perpendicular to the linear arrays, in contrast, would be transmitted to the fatty acid groups which

are compressible. This would produce the thicker secondary linear arrays that were observed.

The model can also account for the formation of cords. The data imply that cords are formed by beads adhering to one another by oriented hydrophobic interactions. The model predicts that beads would adhere to one another most strongly when their linear arrays of TDM are parallel. Adhesion of beads in any other orientation would increase exposure of hydrophobic domains to water and create surface tension which would induce a rotation and realignment. Focal surface excess of TDM or other irregularities in the surface coating could result in hydrophobic adhesive sites at other or irregular intervals on the beads. This a plausible explanation for the irregularity of cords observed in preparations with surface excess of TDM and the adhesion of such beads to plastic surfaces.

Evidence that the molecules of TDM were, in fact, arranged in a circular pattern on beads was obtained by SEM. Circumferential structures were observed for every preparation of TDM-coated beads. The resolution was not sufficient to resolve individual molecules of TDM. Nevertheless, circular structures were seen at every magnification: the higher the magnification, the finer the linear pattern observed. Several lines of evidence suggest that these circles and poles are not artifacts of preparation. First, they were not observed in association with beads that were not coated with TDM. Second, ring structures can be produced by air drying. They are not characteristic of critical-point drying. Finally, the aggregates of material that we call poles were without exception located in the center of the circles, suggesting that they were part of an organized pattern. The lines seen by SEM are 50 to 100 nm in width and probably represent the accumulations of TDM in linear arrays. The lines seen on water would represent even greater degrees of piling up of linear structures. The pattern of fine lines coalescing into thicker lines as illustrated in Fig. 3A is consistent with a surface layer that is compressible in one plane and rigid in the perpendicular plane. The SEM studies frequently demonstrated small aggregates of material at the poles of TDM-coated beads, but not in any other position. We believe that these aggregates strongly support our model because they were present at the only point at which the model predicts a discontinuity in linear adhesive structures.

During preparation of the manuscript, we received atomic-force photomicrographs of the surface of BCG with resolution sufficient to observe linear arrays of TDM (6). The micrographs showed linear arrays with a periodicity of 75 Å (1 Å = 0.1 nm). The chemical nature of the material forming the periodic structures was not identified. However, since TDM has been reported to be a major component of the lipids extractable from the surfaces of both *M. tuberculosis* and BCG (9, 21) and the structures observed were both unusual and are almost precisely as predicted by our model, the pictures are strong evidence for the validity of the model. Nevertheless, the surfaces of mycobacteria are much more complex than that of our beads. They contain many components, including other lipids, that almost certainly contribute to the processes. Our studies show some things that TDM can do. Demonstration that it actually does any of these things in mycobacterial infection is lacking. However, the methodology provides new tools for studies of the biophysical role of glycolipids in the pathogenesis of mycobacterial disease.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant AI-28676 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Bloch, H.** 1950. Studies on the virulence of tubercle bacilli: isolation and biological properties of a constituent of virulent organisms. J. Exp. Med. **91**:197–219.
2. **Bloch, H., and H. Noll.** 1953. Studies on the virulence of tubercle bacilli. Variations in the virulence effect elicited by Tween 80 and thiosemicarbazone. Br. J. Exp. Pathol. **97**:1–16.
3. **Bloch, H., and H. Noll.** 1955. Studies on the virulence of tubercle bacilli. The effect of cord factor on murine tuberculosis. Br. J. Exp. Pathol. **36**:8–17.
4. **Cantor, C. R., and P. R. Schimmel.** 1980. The behavior of biological macromolecules, p. 1340–1348. *In* C. R. Cantor and P. R. Schimmel (ed.), Micelles, biophysical chemistry—part III. W. H. Freeman and Co., San Francisco.
5. **Garber, M. A., W. McManus, B. Bennett, and R. L. Hunter.** 1985. Protein casting: a new replication technique for observing fluid surface structures. J. Electron Microsc. Technique **2**:71–72.
6. **Garcia, A. A., W. C. Pettigrew, and J. Graham.** BCG cell imaging using scanning probe microscopy. Scanning Microsc. Int., in press.
7. **Goren, M. B.** 1990. Mycobacterial fatty acid esters of sugars and sulfosugars, p. 363–461. *In* M. Kates (ed.), Handbook of lipid research. Plenum Press, New York.
8. **Goren, M. B., and P. J. Brennan.** 1979. Mycobacterial lipids: chemistry and biologic activities, p. 63–193. *In* G. P. Youmans (ed.), Tuberculosis. W. B. Saunders Co., Philadelphia.
9. **Goren, M. B., and P. J. Brennan.** 1979. Mycobacterial lipids: chemistry and biologic activities, p. 121. *In* G. P. Youmans (ed.), Tuberculosis. W. B. Saunders Co., Philadelphia.
10. **Koch, R.** 1884. Die aetiologie der tuberkulose. Mitt. Gesundheitsamt **2**:1–88.
11. **Lederer, E.** 1979. Cord factor and related synthetic trehalose diesters. Springer Sem. Immunopathol. **2**:133–148.
12. **Lederer, E., G. Lemaire, J. P. Tenu, and J. F. Petit.** 1987. Natural and synthetic trehalose diesters as immunomodulators, p. 155–164. *In* J. A. Majde (ed.), Immunopharmacology of infectious diseases: vaccine adjuvants and modulators of nonspecific resistance. Alan Liss, Inc., New York.
13. **Lemaire, G., J. P. Tenu, J. F. Petit, and E. Lederer.** 1986. Natural and synthetic trehalose diesters as immunomodulators. Med. Res. Rev. **6**:243–274.
14. **Middlebrook, G., R. J. Dubos, and C. H. Pierce.** 1947. Virulence and morphological characteristics of mammalian tubercle bacilli. J. Exp. Med. **86**:175–184.
15. **Noll, H., H. Bloch, J. Asselineau, and E. Lederer.** 1956. The chemical structure of the cord factor of *Mycobacterium tuberculosis*. Biochim. Biophys. Acta **20**:299–318.
16. **Quinn, P. J., R. D. Koynova, L. J. Lis, and B. G. Tenchov.** 1988. Lamellar gel-lamellar liquid crystal phase transition of dipalmitoylphosphatidylcholine multilayers freeze-dried from aqueous trehalose solutions. A real time X-ray diffraction study. Biochim. Biophys. Acta **942**:315–323.
17. **Ram, P., L. Mazzola, and J. H. Prestegard.** 1989. Orientational analysis of micelle-associated trehalose using an NMR-pseudoenergy approach. J. Am. Chem. Soc. **111**:3176–3182.
18. **Retzinger, G. S., S. C. Meredith, R. L. Hunter, K. Takayama, and F. J. Kézdy.** 1982. Identification of the physiologically active state of the mycobacterial glycolipid trehalose 6,6′-dimycolate and the role of fibrinogen in the biologic activities of trehalose 6,6′-dimycolate monolayers. J. Immunol. **129**:735–744.
19. **Retzinger, G. S., S. C. Meredith, K. Takayama, R. L. Hunter, and F. J. Kézdy.** 1981. The role of surface in the biological activities of trehalose 6,6′-dimycolate: surface properties and development of a model system. J. Biol. Chem. **256**:8208–8216.
20. **Saito, R., A. Tanaka, K. Sugiyama, I. Azuma, Y. Yamamura, M. Kato, and M. B. Goren.** 1976. Adjuvant effect of cord factor, a mycobacterial lipid. Infect. Immun. **13**:776–781.

21. **Silva, C. L., S. M. Ekizlerian, and R. A. Fazioli.** 1985. Role of cord factor in the modulation of infection caused by mycobacteria. Am. J. Pathol. **118:**238–247.
22. **Takayama, K., and N. Qureshi.** 1984. Structure and synthesis of lipids, p. 315–344. *In* G. P. Kubica and L. G. Wayne (ed.), The mycobacteria, a sourcebook. Marcel Dekker, Inc., New York.
23. **Vosika, G., C. Giddings, and G. R. Gray.** 1984. Phase I study of intravenous mycobacterial cell wall skeleton and trehalose dimycolate attached to oil droplets. J. Biol. Response Modif. **3:**620–626.
24. **Vosika, G., J. Schmidtke, A. Goldman, R. Parker, E. Ribi, and G. R. Gray.** 1979. Phase I-II study of intralesional immunotherapy with oil attached *Mycobacterium smegmatis* cell wall skeleton and trehalose dimycolate. Cancer Immunol. Immunother. **6:**135–142.
25. **Weissman, G.** 1975. The molecular basis of acute gout, p. 257–266. *In* G. Weissman and R. Claiborne (ed.), Cell membranes biochemistry, cell biology and pathology. H. P. Publishing Co., Inc., New York.
26. **Yarkoni, E., and H. J. Rapp.** 1979. Influence of oil and Tween concentrations on enhanced endotoxin lethality in mice pretreated with emulsified trehalose 6,6′-dimycolate (cord factor). Infect. Immun. **24:**571–572.
27. **Youmans, G. P.** 1979. Biologic activities of mycobacterial cells and cell components, p. 53–54. *In* G. P. Youmans (ed.), Tuberculosis. W. B. Saunders Co., Philadelphia.